High yield isolation of destruxins A, B, D, E, and E-diol, the major depsipeptide derivatives of *Metarhizium anisopliae* by combining lipophilic Sephadex gel chromatography and HSCCC

Karin Eberhart,[1,2] Christoph Seger,[1] Sonja Sturm,[1] Hermann Strasser,[2] Hermann Stuppner[1]

[1]*Institute of Pharmacy, Pharmacognosy, Leopold-Franzens University of Innsbruck, Innrain 52, A-6020 Innsbruck, Austria*
[2]*Institute of Microbiology, Leopold-Franzens University of Innsbruck, Technikerstraße 25, A-6020 Innsbruck, Austria*

INTRODUCTION

Destruxins (dtxs) are cyclic hexadepsipeptides, secreted by the entomopathogenic fungus *Metarhizium anisopilae*. Dtxs have been studied for more than four decades [1]. Besides their role in fungal pathogenicity, a broad range of pharmacological activities e.g. the prevention of osteoblast formation [2], the formation of ion-channels [3], or effects on heart muscle contraction [4] has been reported.

Dtx derivative isolation is usually carried out by using rather time consuming and insufficient column chromatography and/or preparative RP-HPLC protocols. The need to obtain large amounts of pure dtx derivatives for pharmacological testing purposes and as reference substances in analytical assays [5] prompted us to try alternative purification protocols.

RESULTS

A three step purification protocol was established to isolate destruxins from fungal culture broth:

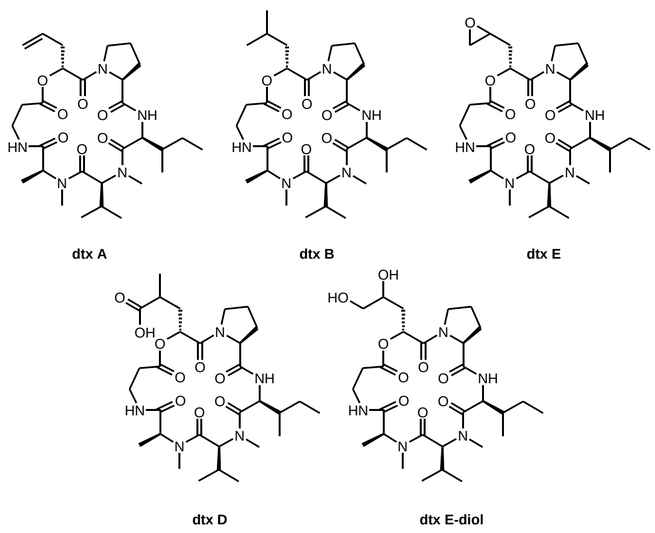
STEP 1: Extraction with dichloromethane (DCM).

STEP 2: Sephadex LH-20 column chromatography with a DCM/acetone step gradient as mobile phase.

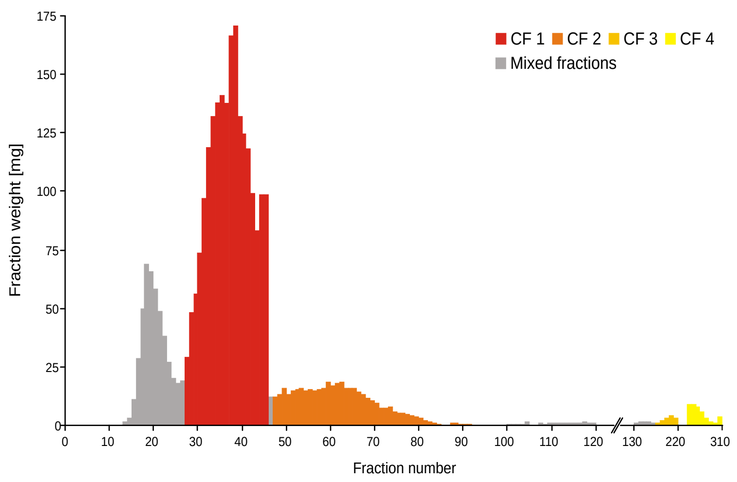
STEP 3: High speed counter current chromatography with a tailored solvent system (PE/EtAc/MeOH/H_2O = 2/5/2/5).

With this setup 519 mg dtx A (68 %), 309 mg dtx B (52 %), 402 mg dtx E (75 %), 22 mg dtx E-diol, and 37 mg dtx D with purities >90% in all cases (% yields from the culture broth) were obtained from a single 10 l submerged fungal culture broth batch.

DESTRUXIN DERIVATIVES

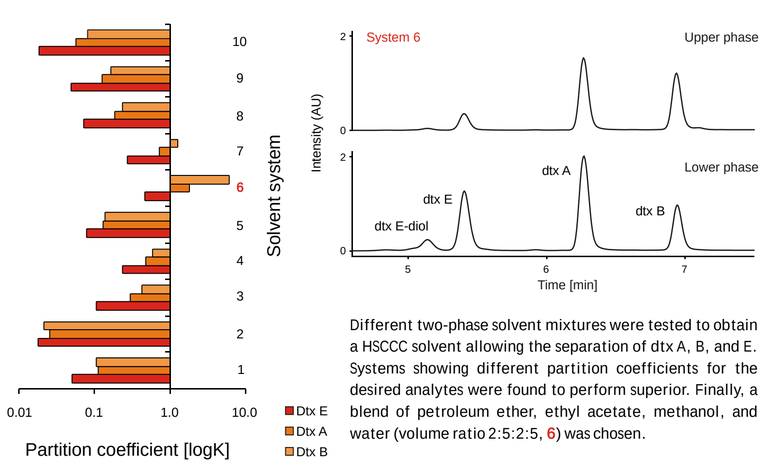


SEPHADEX LH-20



Dtx purification step 2: Fraction profile of Sephadex LH-20 chromatography.

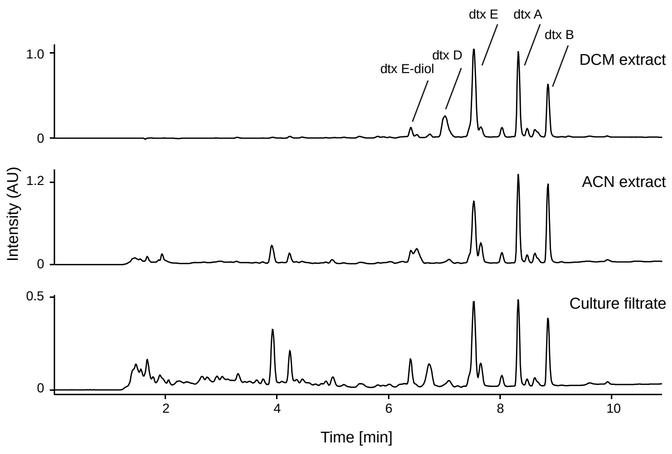
HSCCC OPTIMIZATION



Different two-phase solvent mixtures were tested to obtain a HSCCC solvent allowing the separation of dtx A, B, and E. Systems showing different partition coefficients for the desired analytes were found to perform superior. Finally, a blend of petroleum ether, ethyl acetate, methanol, and water (volume ratio 2:5:2:5, 6) was chosen.

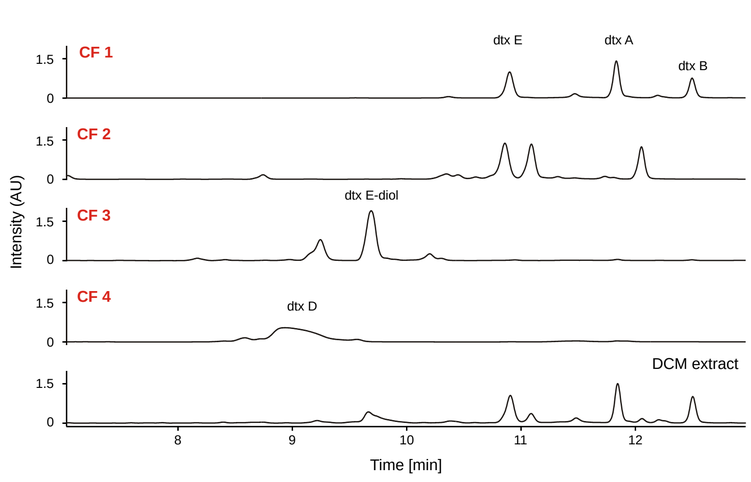
Dtx purification step 3: Optimization of the HSCCC solvent system.

CULTURE FILTRATE WORKUP



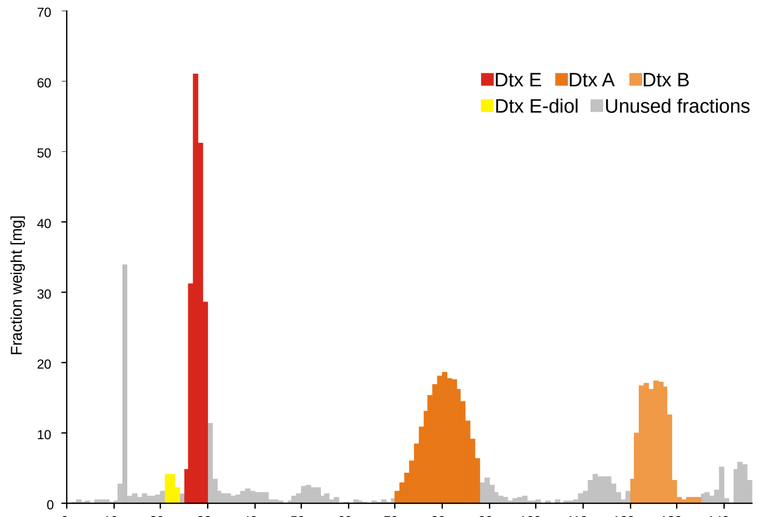
Dtx purification step 1: Extraction of Dtx derivatives from the culture broth.

RESULTS SEPHADEX



Dtx purification step 2: HPLC-DAD chromatograms of combined fractions (CF)

RESULTS HSCCC



Dtx purification step 3: Fraction profile of the HSCCC.

EXPERIMENTAL DETAILS

CULTIVATION CONDITIONS

Metarhizium anisopliae strain BIPESCO5 (KVL275) cultures were grown on S2G medium petri dishes until sporulation. Erlenmeyer flasks containing 500 ml S4G liquid medium were inoculated and incubated at 25°C and 80 % relative humidity on a gyratory shaker (150 rpm) for 5 days. These were used to inoculate a 14 l stirred-tank-reactor (BioEngineering NLF22, Wald, Switzerland) charged with10 l S4G liquid medium (pH 6). The aerated culture (1.6 vvm) ran in batch mode and was stirred (300 rpm) at 23.6°C. After 6 days of growth the culture broth was harvested. The mycelium was separated by filtration through cotton cloth. A final purification of the culture broth was achieved by filtering through a nylon net (20 µm mesh). The culture filtrate was stored at -20 °C until further use.

EXTRACTION

For 1 volume of filtrate 3 volumes of dichloromethane (DCM) were used. The culture filtrate was extracted five times. Combined extracts were washed with 50 ml water and dried with sodium sulphate. After filtration, extracts were evaporated to dryness prior to further use.

SEPHADEX CHROMATOGRAPHY

Sephadex LH-20 media was swollen in acetone/DCM = 15/85. The resulting slurry was used as stationary phase and was equilibrated with DCM prior to use. DCM with increasing amounts of acetone (1 l DCM, 500 ml 85% DCM, 500 ml 50% DCM, 500 ml 0% DCM) at a flow rate of 1.2 ml/min was used as mobile phase. Fractions were collected every 6 ml and evaporated to dryness prior to further use.

HSCCC

HSCCC was carried out using a Model CCC-1000 multilayer coil counter-current chromatograph equipped with a 325 ml coil column and an electronic controller (Pharma-Tech-Research Corp., Baltimore, Maryland, USA). The upper phase was used as stationary phase and the elution mode was head to tail. The effluent was collected in 10 ml fractions, which were subjected directly to HPLC-DAD analysis.

HPLC-DAD/MS

Instrumentation: Agilent HP1100 liquid chromatograph coupled to a Bruker Esquire3000^{plus} ion trap mass spectrometer. Stationary phase: Zorbax SB-C18 (150 mm x 2.0 mm), particle size 3.5 µm. Mobile phase: H_20(A)-ACN(B) at 0.3 ml/min at 23°C. Elution profile: either t= 0 min 5% B, t = 6 min 50 % B, t= 8 min 98 % B, t = 12 min 98 % B; 8 min post time [5] or (accelerated assay) t= 0.0 min 30% B, t = 4.0 min 98 % B, t= 5.3 min 98 % B, t = 5.4 min 30 % B; 4.0 min post time. Detection: UV-DAD at 210 nm. Mass spectrometry parameters: Ion source: ESI, positive mode. Spray voltage: 4500 V. Nebulizer gas: N_2, 40 psi. Dry gas: N_2, 10 l/min 350°C. Scan range: 100-1000 *m/z*.

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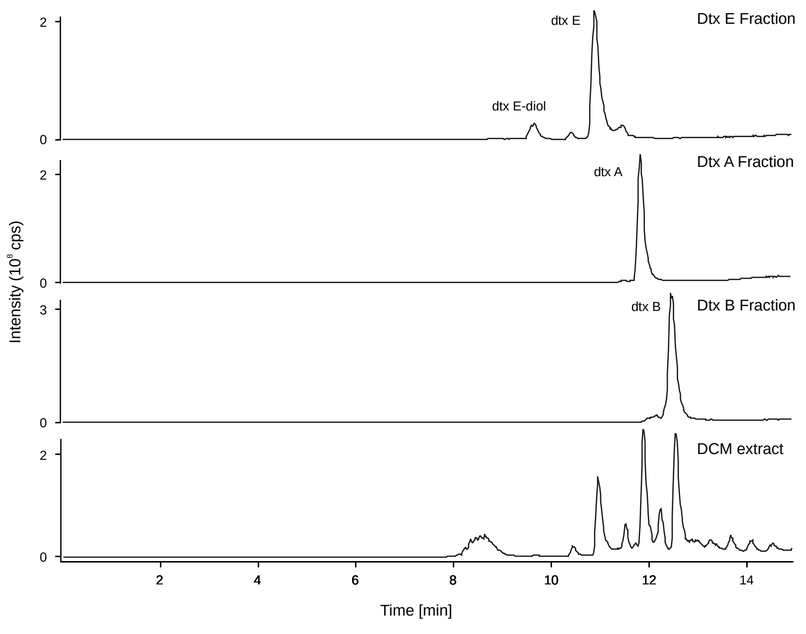
RESULTS HSCCC



Dtx purification step 3: HPLC-DAD/MS derived total ion current chromatograms of the purified Dtx derivatives.

Dtx purification step 2: HPLC-DAD/MS derived chromatograms (UV and selective MS ion traces) of CF1-4.